Uncultivated Tannerella BU045 and BU063 are slim segmented filamentous rods of high prevalence but low abundance in inflammatory disease-associated dental plaques

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Abstract: Uncultivated clones BU045 and BU063 and Tannerella forsythia, a 'consensus periodontal pathogen', are the closest known relatives within the genus Tannerella. They have been described to inhabit different ecological niches of the human oral cavity. In this study, fluorescent in situ hybridization (FISH) and immunofluorescence were combined to investigate the prevalence and abundance of BU045 and BU063 in comparison to T. forsythia in plaques from gingivitis, necrotizing ulcerative gingivitis (NUG) and chronic periodontitis. Phylotype-specific FISH probes identified BU045 and BU063 as elongated thin rods with a segmented structure. Two structurally similar and previously unknown, rare phylotypes (127+ and 997+) were also identified due to partial 16S rRNA sequence identity with T. forsythia. In gingivitis, NUG and periodontitis patients, BU045, BU063, 127+, 997+ and T. forsythia were detected with prevalences of 50/83/71/14 and 81%, 100/100/86/17 and 53%, and 100/100/12/0 and 100%, respectively. Supragingivally, colonization density of all five organisms was generally low, rarely exceeding 0.1% of the total biota. In periodontal pocket samples, however, cell numbers of T. forsythia, but not of the uncultivable phylotypes, were greatly elevated. Our data demonstrate that Tannerella phylotypes BU045, BU063, 127+ and 997+ consist of long slim rods with segments, which, with respect to FISH stainability, often behaved as independent units. The phylotypes are frequent but low-level colonizers of various periodontal disease-associated plaques. Their apparent inability to proliferate to high density seems to exclude any relevance for the pathogenesis of periodontal diseases.

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Uncultivated *Tannerella* BU045 and BU063 are slim segmented filamentous rods of high prevalence but low abundance in disease-associated dental plaques

Running title: Single cell analysis of oral *Tannerella* populations

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Abbreviations: FISH, fluorescent *in situ* hybridization; IF, indirect immunofluorescence; mAb, monoclonal antibody; NUG, necrotizing ulcerative gingivitis

Keywords: 16S rRNA, plaque, fluorescent in situ hybridization, immunofluorescence, subgingival plaque, periodontitis

SUMMARY

Yet unidentified and uncultivated clones BU045 and BU063 and *Tannerella forsythia,* a 'consensus periodontal pathogen', are the closest known relatives within the genus *Tannerella.* Reportedly, they inhabit preferentially different ecologies of the human oral cavity. In this study, fluorescent in situ hybridisation (FISH) and immunofluorescence (IF) were used to investigate the prevalence and abundance of BU045 and BU063 in comparison to *T. forsythia* in plaques from gingivitis, necrotizing ulcerative gingivitis (NUG), and chronic periodontitis. Phylotype-specific FISH probes identified BU045 and BU063 as elongated thin rods with a segmented structure. Two further, structurally similar and so far unknown, rare phylotypes (127^+ and 997^+) were identified due to partial 16S rRNA sequence identity with *T. forsythia*. In gingivitis, NUG, and periodontitis patients, BU045, BU063, 127^+, 997^+ and *T. forsythia* were detected with prevalences of 50/83/71/14 and 81%, 100/100/86/17 and 53%, and 100/100/12/0 and 100%, respectively. Supragingivally, colonisation density of all five organisms was generally low, rarely exceeding 0.1% of the total biota. However, in periodontal pocket samples *T. forsythia* cell numbers were greatly elevated, in contrast to any of the uncultivable phylotypes. Our data demonstrate that *Tannerella* phyloptypes BU045, BU063, 127^+ consist of long slim segmented rods that are frequent but low-level colonizers of various periodontal disease-associated plaques. Their apparent inability to proliferate to high density, which seems to exclude any relevance to periodontal diseases, could depend on distinct nutritional requirements but might as well be related to their peculiar elongated and segmented structure.

INTRODUCTION

Relying on comprehensive culture analyses Moore and Moore {%Moore and Moore, 1994, Periodontol. 2000, 5, 66-77} estimated that the human oral cavity might harbour some 500 bacterial species. With the emergence of culture independent methods for the identification of microbial biota {Amann et al., 1995, Microbiol. Rev., 59, 143-169} it became clear that probably less than 50% of the oral taxa have been cultured so far {Wilson et al., 1997, Reviews Med Microbiol, 8, 91-101}. During the last decade hundreds of previously unknown phylotypes and clones have been identified in studies investigating 16S rRNA diversity in different forms of dental plaque {Paster et al., 2001, J. Bacteriol., 183, 3770-3783; Paster et al., 2002, Ann Periodontol, 7, 8-16; de Lillo et al., 2006, Oral Microbiol Immunol, 21, 61-8; Munson et al., 2004, J. Clin. Microbiol., 42, 3023-3029; Aas et al., 2005, J. Clin. Microbiol., 43, 5721-5732; Kumar et al., 2005, J. Clin. Microbiol., 43, 3944-3955}. Among the many uncultivable human oral phylotypes two clones with an apparently supragingival habitat, BU045 and BU063, caught some interest, because their 16S rRNA gene sequence groups them within the genus *Tannerella* {Paster et al., 2001, J. Bacteriol., 183, 3770-3783; Leys et al., 2002, J. Clin. Microbiol., 40, 821-825; de Lillo et al., 2004, J Clin Microbiol, 42, 5523-5527}, which otherwise contains, besides some uncultivated clones from soil, only a single species, *Tannerella forsythia* {Tanner et al., 1986, Int. J. Syst. Bacteriol., 36, 213-221; Tanner and Izard, 2006, Periodontology 2000, 42, 88-113}. *T. forsythia* is a 'consensus periodontal pathogen' {Haffajee and Socransky, 2006, Periodontology 2000, 42, 7-12} and associated with subgingival plaque from chronic and occasionally aggressive periodontitis {Tanner and Izard, 2006, Periodontology 2000, 42, 88-113 \for review} rather than periodontal health as the two clones BU045 and BU063. Although all three taxa colonize areas of the gums in the human oral cavity, they home in quite distinct environments with markedly different nutrient sources.

The aims of the present study were to identify the uncultivable *Tannerella* phylotypes represented by clones BU045 and BU063, and to compare with *T. forsythia* their prevalence

and abundance in plaques collected from patients with gingivitis, necrotizing ulcerative gingivitis (NUG) and chronic progressive periodontitis. Based on the available infrastructure, experience and DNA sequence information, we opted for a microscopic approach combining fluorescent in situ hybridization (FISH), using DNA probes to phylotype-specific 16S rRNA sequences, with indirect immunofluoresence (IF) to target the *Tannerella* organisms.

METHODS

Strains, dental plaque samples and cultivation. Strains of oral bacteria were grown anaerobically (85% N_2, 10% H_2, 5% CO_2) at 37 °C in fluid universal medium (FUM) {Gmür and Guggenheim, 1983, Infect. Immun., 42, 459-470} enriched with 5% horse serum. They were harvested after 24-36 hours from the log-phase of growth.

Subgingival plaque samples (n=17) were obtained with paper points {Gmür et al., 1989, J. Periodont. Res., 24, 113-120} from deep periodontal pockets of seven patients (five female) with a mean age of 59.1 years (range 50-71 years). The patients had been referred to our institute for microbial testing due to refractory chronic periodontitis {Armitage, 1999, Ann. Periodontol., 4, 1-6} at the sampled sites. Samples were processed for culture, FISH, and IF as described {Gmür and Thurnheer, 2002, Microbiology, 148, 1379-1387}.

Marginal supragingival plaque samples derived from Chinese patients affected by gingivitis (n=7) or necrotizing ulcerative gingivitis (NUG) (n=7). These samples were selected from a collection of frozen samples described in a previous study {Gmür et al., 2004, Eur. J. Oral Sci., 112, 33-41}. They were pooled samples (one sample per patient with plaque collected from several teeth) from gingivitis (four female) and NUG patients (four female) with a mean age of 35.9 years (range 27-53 years) and 32.6 years (range 27-52 years), respectively.

Preparation of multiwell slides for IF and FISH. For IF and FISH of cultured bacteria, strains were prepared as described {Gmür and Thurnheer, 2002, Microbiology, 148, 1379-

1387}. Cell suspensions were used fresh or after storage at -20 °C. Briefly, fresh cell suspensions were spotted directly on 18- or 24-well slides (Cel-Line Associates), air-dried and fixed by a 20-min incubation at 4 °C in 4% paraformaldehyde/PBS (FISH) or by a 2-min at exposure room temperature to methanol (IF). To obtain a bank of frozen cell suspensions, freshly harvested cells were pelleted by centrifugation at 13'000 x g, resuspended for 20 min in 3.6% paraformaldehyde/PBS (4 °C), re-centrifuged and then stored until further use in 50% ethanol at -20 °C. Plaque samples were vortexed for 60 s at maximum speed and immediately diluted 1:8 in coating buffer (0.9% NaCl, 0.02% NaN_3, 0.00025% cetyltrimethylammonium bromide). Thereafter, 10 µl of plaque suspension per well was dropped onto 18- or 24-well slides, air-dried and fixed as described above. Slides, stored at room temperature, were processed for FISH or IF within 48 hours from fixation.

FISH and IF procedures. FISH: Custom-synthesized oligonucleotide probes, labelled at the 5'-end with Cy3 or 6-FAM, were purchased from Microsynth. Probes were designed according to the criteria described by Manz {%Manz, 1999, Methods Enzymol., 310, 79-91} using the ARB software {Ludwig et al., 2004, Nucleic Acids Res., 32, 1363-1371} (http://www.arb-home.de) and rRNA sequence information from 'The Ribosomal Data Base Project II' {Cole et al., 2005, Nucleic Acids Res., 33 , D294-D296} (http://rdp.cme.msu.edu/) and the 'National Center for Biotechnology Information' (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of the employed probes for *Bacteria*, *T. forsythia* and the *Tannerella* clones BU045 and BU063 are listed in Table 1. Probe EUB338 was used as universal positive control, but results are not further detailed below. Probes were used at final concentrations of 5 ng μl^{-1} (Cy-3 conjugates) and 15 ng μl^{-1} (FAM-conjugates) in the presence of 40% formamide in the hybridization buffer. FISH was performed in 50 ml plastic centrifuge tubes at 46 °C as described {Thurnheer et al., 2001, J. Microbiol. Methods, 44, 39-47} except for the following modifications {Gmür and Lüthi-Schaller, 2007, J. Microbiol. Meth., 69, 402-405}: (i) slides were not dehydrated in ethanol prior hybridization, (ii) wells were covered for 60 min at 37 °C with Denhardt's solution (D-2532, Sigma-Aldrich; diluted 1:50 in 0.9% NaCl) in the presence protectRNA™ RNase

inhibitor (R-7397, Sigma-Aldrich, diluted 1:500 in 0.9% NaCl) prior hybridization to prevent unspecific binding of the probes to the bacterial surface, (iii) reagent volumes for 4-mm-wells were 3–5 µl, and (iv) hybridization was limited to 120 min. *Immunofluorescence: T. forsythia* was stained with monoclonal antibody (mAb) 116BF1.2 {Werner-Felmayer et al., 1988, J. Dent. Res., 67, 548-553}. mAb-binding was visualized by consecutive incubations with biotinylated goat anti-mouse IgG (Sigma) and avidin-fluorescein isothiocyanate (FITC) (Sigma), diluted 1:250 and 1:1500, respectively, in borate-buffered saline supplemented with 0.5% skim-milk powder and 0.05% Tween 20 {Werner-Felmayer et al., 1988, J. Dent. Res., 67, 548-553}.

Combined IF and FISH: The combined application of the two assays was performed using a recently described experimental protocol {Gmür and Lüthi-Schaller, 2007, J. Microbiol. Meth., in press}. Cells were visualized with an Olympus BX60 epifluorescence microscope [Olympus Optical (Schweiz)] equipped with phase-contrast, an HBO 103 W/2 mercury photo optic lamp (Osram) andnOlympus Filter sets U-MNIBA (6-FAM, FITC), U-MA41007 (Cy3) and BX-DFC5 (6-FAM/FITC/Cy3). Color micrographs were taken with a digital Olympus Camedia 3030 camera, transferred to an iMac G5 personal computer and processed using iPhoto 6.0.4 (Apple) and Photoshop 6.0 (Adobe) without any qualitative changes to the raw images. The lower detection limit of both assays was 3 x 10^3 for a typical sample containing approximately 10^8 bacteria {Gmür, 1995, Value of new serological probes for the study of putative…}.

Estimation of total bacterial cell numbers: The total cell number of samples from the Chinese gingivitis and NUG patients had been determined in a previous study by automated image analysis of fluorescence labelled cells {Gmür et al., 2004, Eur. J. Oral Sci., 112, 33-41}. Cell numbers present in periodontal pocket samples were determined by anaerobic cultivation for 5 days (37 °C) on Columbia blood agar (Oxoid) supplemented with 5% haemolysed human blood. In a previous study we have shown that numbers of fluorescent cells determined by visual microscopy and by image analysis correspond well, whereas total numbers of CFU are about a factor of 3 lower than those determined by image analysis

{Gmür et al., 2000, Eur. J. Oral Sci., 108, 393-402}. Therefore, we multiplied the numbers of CFU by 3 to generate the total cell number estimates used to calculate the phylotype proportions shown in Fig. 2.

Measurements of bacterial dimensions: The length of FISH-labelled *Tannerella* organisms was determined by assessing randomly selected cells present on digital images with the imaging software cell^P 2.3 (build 1121) (Olympus). Images were made at 1000x magnification with an Olympus BX61 epifluorescence microscope (Olympus) equipped with phase-contrast, an HBO 103 W/2 mercury photo optic lamp (Osram), Chroma Filter sets 31001 (6-FAM, FITC), 41007a (Cy3) and 51004v2 (6-FAM/FITC/Cy3) (Chroma Technology Corporation, Rockingham, VT, USA), and a F-View II Digital B/W Camera (Olympus).

RESULTS

Morphology of BU045, BU063, and *T. forsythia*.

Probes Tan1260a and Tan1260b, designed to detect *Tannerella* clones BU045 and BU063, respectively, were negative with all 13 *T. forsythia* strains tested and so far did not label cultivable strains form other taxa. Applied to subgingival and supragingival plaque samples the two probes identified straight or irregularly bent, thin rods with pointed ends. Positive cells were found both planktonically and trapped in dense aggregates (Fig. 1 A-C). The rods displayed a segmented structure. Quite frequently intensively stained segments were adjacent to terminal or inner segments without any FISH staining (Fig. 1 E-H). Measurements with two samples from different periodontitis patients showed that the length of BU045 and B063 cells ranged broadly (Table 2). Moreover, sample IB (26d) contained on average longer BU045 and B063 cells than sample SB (26d).

All 13 cultured *T. forsythia* strains were strongly positive with FISH probes Tfor127, Tfor439, Tfor582, and Tfor997, but not with Tan1260a or Tan1260b. In subgingival and supragingival

plaques Tfor439 and Tfor582 stained always slightly spindle-shaped rods (Fig. 1 I and J) that were much shorter than BU045 and BU063 (Table 2) and frequently colonized with high density large multi-species aggregates (Fig. 1 D). Double-labelling experiments with Cy3- and FAM-tagged probes showed as expected that the four probes for *T. forsythia* recognized the same cells (data not shown). Analogously, combined labelling by immunofluorescence with the *T. forsythia*-specific mAb 116BF1.2 and FISH using any of the six *Tannerella* probes, demonstrated that the antibody and DNA probes Tfor127, Tfor439, Tfor582, and Tfor997 identified the same short spindle-shaped cells {\data not shown, for a representative image see \Gmür and Lüthi-Schaller, 2007, J. Microbiol. Meth., 69, 402-405}. We never found any 116BF1.2-positive elongated cells. Unexpectedly, probes Tfor127 and Tfor997 detected two further rare populations of very elongated segmented rods (Fig. 1 K). These thin cells were not stained by any other of the six *Tannerella* probes, suggesting that they neither belong to *T. forsythia senso stricto,* nor to clones BU045 or BU063. They are designated in the following as phylotypes 127^{+} and 997^{+}, whereby the numbers refer to the DNA probes used for their detection.

Fig. 2 describes prevalence and abundance of these four so far uncultivable phylotypes and of *T. forsythia* in samples from gingivitis, NUG, and chronic periodontitis patients. The gingivitis and NUG samples contained on average total cell numbers of 2.6×10^{8} ml^{-1} (range 4×10^{7} - 7.4×10^{8}) and 7.6×10^{8} ml^{-1} (range 2.9×10^{8} - 1.4×10^{9}) whereas the periodontitis samples yielded on average 1.7×10^{7} ml^{-1} CFU (range 4.4×10^{6} - 5.8×10^{7}) (data not shown). Read column-by-column, Fig. 2 shows that clone BU045 was identified by Tan1260a in gingivitis, NUG and periodontitis samples with a prevalence of 50, 83, and 81%, and clone BU063 (Tan1260b) with 100%, 100%, and 53%, respectively. Both clones accounted in most samples for less than 0.1% of the total biota; in only two cases BU063 exceeded 1%. *T. forsythia* was detected with a 100% prevalence in supragingival gingivitis and NUG plaques by three of the four rRNA probes and by the mAb. However, cell densities were consistently very low and often near the detection limit of these microscopic assays (approximately 0.3×10^{4} ml^{-1}). In subgingival plaques from deep periodontal pockets *T. forsythia* prevalence was

again 100%, but now all but three samples contained high levels of the organism. In more than half of the samples *T. forsythia* exceeded 10% of the total biota. The IF assay almost always detected higher *T. forsythia* cell numbers than FISH. The two new phylotypes 127^+ and 997^+ never reached high densities. Phylotype 997^+ was very rare, whereas 127^+ occurred regularly in supragingival plaques, showing a prevalence of 71%, 86%, and 12% in gingivitis, NUG, and periodontitis samples, respectively.

DISCUSSION

This study has demonstrated that four uncultivated *Tannerella* phylotypes consist of slim, elongated, segmented rods with one or two tapered ends. Among them are the two previously described clones BU045 and BU063 {Paster et al., 2001, J. Bacteriol., 183, 3770-3783; Leys et al., 2002, J. Clin. Microbiol., 40, 821-825; de Lillo et al., 2004, J Clin Microbiol, 42, 5523-5527} found here in supra- and subgingival from patients with gingivitis, NUG, or chronic periodontitis with prevalences between 50 and 100%. The two other phylotypes are previously unknown taxa, tentatively designated 127+ and 997+ due two their sequence homology with *T. forsythia* in regions 127-146 and 997-1014, respectively, of the 16S rRNA gene. Their prevalence in periodontal pocket samples is very low, but at least 127+ has revealed high prevalence in the two groups of Chinese gingivitis and NUG samples. In contrast to *T. forsythia*, these uncultivable phylotypes seem, however, to proliferate never to high densities in supra- or subgingival plaque and therefore are unlikely to be of any importance in the pathogenesis of these periodontal diseases.

Our data on the prevalence and abundance of *T. forsythia* in the periodontal pocket microbiota compare well with earlier results from many different studies {Tanner and Izard, 2006, Periodontology 2000, 42, 88-\for review\}. With respect to supragingival plaques from gingivitis and NUG patients the low abundance of *T. forsythia* had to be expected based on

previous findings with supragingival plaque, albeit from quite different study groups {Gmür and Guggenheim, 1994, J. Dent. Res., 73, 1421-1428; Paster et al., 2002, Ann Periodontol, 7, 8-16}. However, the observation of a 100% prevalence in these samples is remarkable as it shows that *T. forsythia* is even supragingivally an ubiquitous organism in people affected by severe gingival inflammation. The critical unanswered question remains, however, what causes *T. forsythia* to proliferate to several orders of magnitude higher cell densities when they reach the environment of the periodontal pocket? It should be mentioned that our prevalence scores for supragingival plaques are much higher than those reported by Socransky and Haffajee {%Socransky and Haffajee, 2002, Periodontol 2000, 28, 12-55} for healthy subjects and for patients with periodontitis. However, these authors studied not directly comparable patient groups, too, and they used the checkerboard assay with whole genomic probe hybridization, which in terms of assay sensitivity has never been compared to our procedures.

The unexpected finding that cells of the four uncultivated *Tannerella* phylotypes are thin segmented filamentous rods is interesting. Morphologically comparable cell types have been described as segmented filamentous bacteria or SFB {Davis and Savage, 1974, Infect Immun, 10, 948-56}. SFB live in the small intestine of arthropods, fish, birds and many mammalian species including humans, where they are anchored to the epithelial surface {Tannock et al., 1984, Appl Environ Microbiol, 47, 441-442; Smith, 1997, J Comp Pathol, 117, 185-90; Meyerholz et al., 2002, Infect Immun, 70, 3277-80}. They are considered to be non-cultivable, species-specific, non-pathogenic, and potent activators of the mucosal immune system. However, based on the available 16S RNA gene sequence data, the *Tannerella* phylotypes and the SFB belong to quite distinct phylogenic groups {Snel et al., 1995, Int J Syst Bacteriol, 45, 780-782; Tanner and Izard, 2006, Periodontology 2000, 42, 88-113} and labelling of any SFB by our FISH probes can be exluded. The length of cells from the uncultivable *Tannerella* phylotypes was quite variable. It is our impression that the variable number of segments per cell could explain length variation, however, this hypothesis will need to be verified by electron microscopy. In contrast, *T. forsythia* cells were

consistently short, 2.3 µm on average, with no evidence for segmentation. Occasionally, individual segments of apparently normal cells of BU045, BU063, 127+, and 997+ were not stainable by FISH. Estimated from such unstained segments, the length of individual segments is similar to the one of a single *T. forsythia* cell. The biological significance of such cell segmentation in the genus *Tannerella* remains to be elucidated. Lack of FISH staining of a single segment of a multi-segmented could reflect structural differences in cell wall composition resulting in greatly reduced probe permeability in affected segments, or, perhaps more likely, indicate that such segments contain no or much less rRNA than directly adjacent segments. This would imply that these structures function and die as individual self-sustaining units. It would be interesting to test whether individual segments could give rise to new multi-segmented cells, however, so far our attempts to cultivated BU045 and BU063 have not progressed beyond the stage of heterogenous enrichment cultures (C. Wyss, personal communication).

Obtained with a procedure developed for the purpose of this study {Gmür and Lüthi-Schaller, 2007, J. Microbiol. Meth., 69, 402-405}, our combined FISH-IF experiments clearly demonstrated that the four FISH probes to *T. forsythia* and mAb 116BF1.2 (and 103BF1.1, data not shown) all identify specifically the same short rods with tapered ends. This again confirms the specificity for *T. forsythia* of antibody 116BF1.2, used since 1989 in many studies to detect and enumerate this periodontitis associated microorganism {Gmür et al., 1989, J. Periodont. Res., 24, 113-120; Gmür and Guggenheim, 1994, J. Dent. Res., 73, 1421-1428; Gmür et al., 1999, Eur. J. Oral Sci., 107, 225-228; Kamma et al., 2004, Oral Microbiol. Immunol., 19, 314-321}. Notably, cells detected in plaque were usually shorter than cells derived from in vitro grown *T. forsythia* strains (data not shown). The morphology of the identified *T. forsythia* cells is in agreement with earlier descriptions by Tanner et al. {%Tanner et al., 1986, Int. J. Syst. Bacteriol., 36, 213-221} and Lai et al. {%Lai et al., 1987, Oral Microbiol. Immunol., 2, 152-157}, but contrasts with the description by Gersdorf et al. {%Gersdorf et al., 1993, J. Clin. Microbiol., 31, 941-946}, who considered *T. forsythia* to be elongated thin cells, reminiscent of the uncultivable cells identified in this study. The latter

study used the 16S rRNA FISH probe BFV530 for detection, a probe that with the rRNA gene sequences available nowadays can be shown to hybridise to *T. forsythia* and to BU045 and BU063. Thus most probably Gersdorf et al. {%Gersdorf et al., 1993, J. Clin. Microbiol., 31, 941-946} had not observed *T. forsythia* but BU045 and/or BU063, and this several years before they were (re)detected by partial sequencing of plaque-derived 16S rRNA clones {Paster et al., 2001, J. Bacteriol., 183, 3770-3783}.

Table 1. rRNA probe sequences, target sites and target taxa

Probe	Sequence (5' to 3')	Site	Target	Source*
EUB338	GCTGCCTCCCGTAGGAGT	338-55	Most eubacteria	1,2
Tan1260a	CGCATCCGATCACTCGGT	1260-78	*Tannerella* Clone BU045	3
Tan1260b	TGCATCCGATCGCTCGGT	1260-78	*Tannerella* Clone BU063	3
Tfor127	CTCTGTTGCGGGCAGGTTAC	127-46	*T. forsythia*	3
Tfor439	CGTATCTCATTTTATTCCCCTGTA	439-62	*T. forsythia*	4
Tfor582	GCGGACTTAACAGCCCACCT	582-601	*T. forsythia*	5
Tfor997	TCACTCTCCGTCGTCTAC	997-1014	*T. forsythia*	3

* 1, {Amann et al., 1995, Microbiol. Rev., 59, 143-169}; 2, {Daims et al., 1999, System. Appl. Microbiol., 22, 434-444}; 3, this study; 4, {Sunde et al., 2003, Microbiology, 149, 1095-1102}; 5, {Gmür and Lüthi-Schaller, 2007, J. Microbiol. Meth., 69, 402-405}

Table 2. Length of BU045, BU063 and *T. forsythia* bacteria in two subgingival periodontitis samples

	Bacteria stained by FISH with 16S rRNA probes					
Sample	Tan1260a	Tan1260b	Tfor127	Tfor439	Tfor582	Tfor997
IB (26d)	8.0±4.5 (32)* 2.2-26.8	15.9±7.0 (39) 4.1-28.9	2.4±0.8 (53) 1.1-4.2	nt†	nt	nt
SB (26d)	4.9±2.5 (33) 2.1-11.3	9.6±4.4 (16) 3.4-20.5	2.2±0.5 (170) 0.8-4.0	2.2±0.5 (375) 1.0-4.7	2.3±0.6 (261) 0.8-5.7	2.1±0.5 (259) 1.0-4.1

* Mean ± standard deviation and range of cell length [µm]. The number of measured cells is given in parentheses.

† nt, not determined

Figure legend

Fig 1. Morphology of bacteria labeled in plaque samples by FISH with 16S rRNA probes to *Tannerella* group organisms. (A and B) Phase contrast and epifluorescence micrographs of a representative field of periodontitis plaque IB (26d) stained with Tan1260a-Cy3 and Tan1260b-FAM. BU045 (orange-red) and BU063 (green) cells are identified as elongated thin rods with pointed ends. (C) Tan1260a-Cy3 stained BU045 and Tan1260b-FAM stained BU065 cells in and around a large bacterial aggregate of supragingival plaque (NUG35). We never observed bacteria of the two phylotypes reach higher densities than in this field. (D) Similar aggregate from subgingival plaque PF (48b) stained with Tfor439-Cy3 for *T. forsythia.* Such focal high-density colonization by *T. forsythia* was restricted subgingival plaques from refractory chronic periodontitis. Note the difference in cell length in comparison to image C. (E and F) Same plaque and same staining as in images B. BU045 and BU063 cells marked by blue rectangles and arrows reveal a segmented structure with internal or terminal segments completely unlabeled by FISH. (G and H) Magnification of the red-circled area in image E, illustrating the segmentation of a BU045 cell. (I, J) Area of a periodontitis plaque (DG 16d) stained simultaneously for *T. forsythia* by IF(green) and FISH (red). Clearly, mAb 116BF1.2 and probe Tfor582-Cy3 detect the same cells. (K, L) Phase contrast and epifluorescence images of a representative field from sample NUG35 stained with Tfor439-Cy3, showing the characteristic morphotype (short rods with pointed ends) of *T. forsythia* in plaque. (M) Another image of plaque NUG35, now stained with Tfor127-Cy3. Besides a cell with the standard morphology of *T. forsythia* (arrow), two much longer segmented cells are positive. These elongated cells were not labelled any of the other probes, nor by mAb 116BF1.2, which indicates that they represent a further previously unknown *Tannerella* phylotype. Bars, 10 µm.

Fig. 2. Grid plot describing the prevalence and abundance of FISH- or mAb-labelled *Tannerella* populations in supragingival plaque samples from Chinese patients with NUG or gingivitis, and in subgingival plaque samples from Swiss patients with refractory chronic periodontitis. *Tannerella* phylotypes and *T. forsythia* were detected by FISH probes to specific 16S rRNA sequences and by expression of a *T. forsythia*-specific surface antigen (last column). Numbers in fields indicate counted cell numbers (x 10^4) ml^{-1}. Key to the color-code of fields: white with nt, not tested; white with –, no stained bacteria detected; grey, ≤ 0.1% of all bacteria; green, between 0.1% and 1% of all bacteria; yellow, 1% - 10% of all bacteria; red, > 10% of all bacteria.

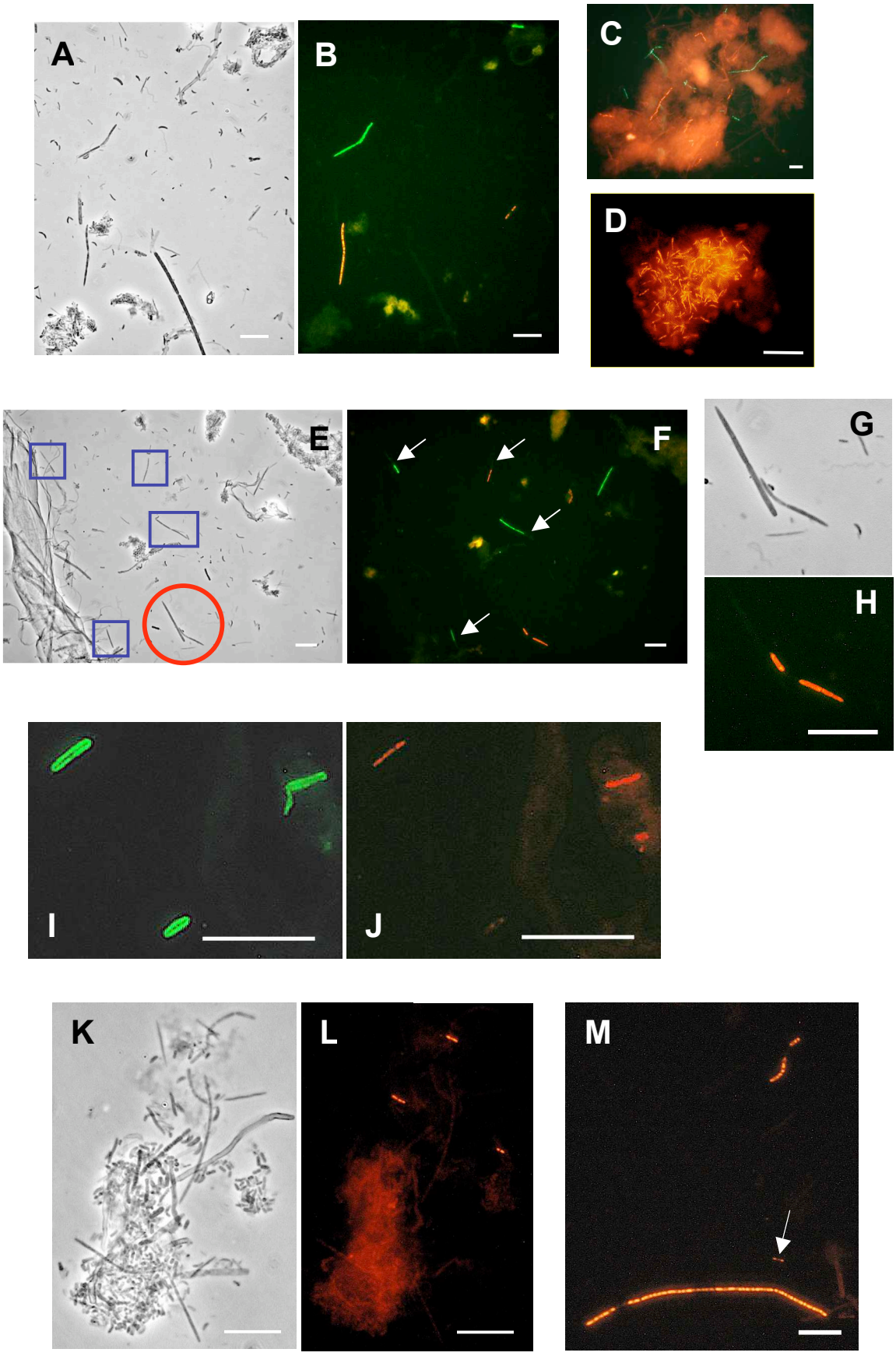

Fig. 1

Disease	Plaque	FISH with probe								IF with mAb
		Tan1260a	Tan1260b	Tfor127		Tfor439	Tfor582	Tfor997		116BF1.2
				long cells	short cells	short cells	short cells	long cells	short cells	short cells
Gingivitis	G3	-	2*	-	-	nt	nt	-	-	26
	G10	nt	nt	0.4	4	0.2	nt	-	2	53
	G12	1.0	2	0.2	15	10	10	-	7	62
	G16	-	15	-	3	3	3	-	3	71
	G18	-	2	0.2	3	1.2	-	-	2	52
	G20	17	30	17	1.1	1.3	1.0	0.2	1.4	3
	G21	5	174	38	13	1.3	3	-	3	6
NUG	NUG33	4	87	12	14	4	3		4	47
	NUG35	23	37	33	49	15	9	13	94	150
	NUG37	0.8	15	6	6	5	5	-	5	210
	NUG38	-	26	2	0.4	1.2	-	-	0.8	8
	NUG39	0.2	53	0.8	0.4	0.4	-	-	2	60
	NUG43	0.8	106	0.6	8	8	8	-	5	130
	NUG49	nt	nt	-	0.4	-	-	nt	nt	26
Periodontitis	PF (17m)	-	132	-	1320	1320	1320	nt	nt	1584
	PF (48b)	0.7	0.2	-	358	80	325	nt	nt	1584
	HA (17p)	1.0	0.1	0.3	3	2	1.1	-	0.7	339
	HA (25m)	4	-	0.2	173	678	255	-	271	2931
	HA (47d)	-	-	-	1.1	0.7	1.4	-	1.4	323
	CR (17m)	0	2	-	133	135	109	-	142	276
	CR (23d)	0.5	-	-	5	6	5	-	6	106
	CR (47d)	1.3	6	-	36	38	27	-	33	162
	DG(16d)	3	-	-	337	84	1089	-	99	1861
	DG (37d)	-	-	-	1271	1320	630	-	1881	124
	SB (26d)	23	2	-	2904	1985	2640	-	334	3476
	IB (18m)	15	32	-	766	586	564	-	421	528
	IB (26d)	57	47	-	244	255	384	-	276	348
	IB (36d)	0.2	-	-	739	732	541	-	899	132
	HP (11m)	-	-	-	440	469	464	-	420	660
	HP (22d)	0.2	-	-	333	446	485	-	327	662
	HP (36d)	2	1.1	-	710	716	535	-	779	1267
		***Tannerella* BU045**	***Tannerella* BU063**	**New *Tannerella* phylotype**	***Tannerella forsythia***	***Tannerella forsythia***	***Tannerella forsythia***	**New *Tannerella* phylotype**	***Tannerella forsythia***	***Tannerella forsythia***

* all numbers in fields indicated the observed cell number x 10^4 per ml of sample suspension, e.g. 244 = 2.44 x 10^6 ml^{-1}

% of total bacterial cell number or CFU	≤ 0.1%	0.1 bis 1%	1%-10%	>10%

Fig. 2